Abundant and Diverse Fungal Microbiota in the Murine Intestine

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Enteric microbiota play a variety of roles in intestinal health and disease. While bacteria in the intestine have been broadly characterized, little is known about the abundance or diversity of enteric fungi. This study utilized a culture-independent method termed oligonucleotide fingerprinting of rRNA genes (OFRG) to describe the compositions of fungal and bacterial rRNA genes from small and large intestines (tissue and luminal contents) of restricted-flora and specific-pathogen-free mice. OFRG analysis identified rRNA genes from all four major fungal phyla: Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota. The largest assemblages of fungal rRNA sequences were related to the genera Acremonium, Monilinia, Fusarium, Cryptococcus/Filobasidium, Sclerotderma, Catenomycyes, Spizellomyces, Neocallimastix, Powellomyces, Entophlyctes, Mortierella, and Smittium and the order Mucorales. The majority of bacterial rRNA gene clones were affiliated with the taxa Bacteroidetes, Firmicutes, Acinetobacter, and Lactobacillus. Sequence-selective PCR analyses also detected several of these bacterial and fungal rRNA genes in the mouse chow. Fluorescence in situ hybridization analysis with a fungal small-subunit rRNA probe revealed morphologically diverse microorganisms resident in the mucus biofilm adjacent to the cecal and proximal colonic epithelium. Hybridizing organisms comprised about 2% of the DAPI and proximal colonic epithelium. Hybridizing organisms comprised about 2% of the DAPI

Gut microflora play a variety of roles in health and disease. Some bacteria synthesize nutrients utilized by the host, while others transform dietary substances into carcinogens (8). Standard indigenous bacteria appear to be involved in both the development of a normal gut immune system and, in the case of inflammatory bowel disease, the induction of inappropriate inflammatory responses (8, 41, 50, 57). In addition, specific intestinal microbial populations in infants have been correlated with increased incidence of atopy (9, 30). To better understand the roles that specific organisms or consortia of organisms play in such functions, investigators will need a variety of experimental capabilities, not the least of which is the ability to identify the microorganisms inhabiting the gut.

Obtaining detailed descriptions of microbial community composition is a continuing challenge. Microorganisms have been traditionally identified through characterization of their morphological and physiological traits. However, this approach is limited by its reliance on culture techniques, which detect only a fraction of microorganisms (5, 33, 54). While the development of strategies to directly analyze rRNA molecules and genes from environmental samples has provided a means to examine microbial communities without the culture bias, most of these methods, including denaturing gradient gel electrophoresis (42), terminal restriction fragment length polymorphisms (38), ribosomal intergenic spacer analysis (11), and amplified ribosomal DNA restriction analysis (61), do not typically generate detailed descriptions of microbial communities. Nucleotide sequence analysis of rRNA gene clone libraries can be used to facilitate thorough depictions of microbial composition, yet this approach is usually impractical because of the high costs associated with examining large numbers of samples from complex communities such as those found in the gut.

In this study, we used an array-based method termed oligonucleotide fingerprinting of rRNA genes (OFRG) (10, 58, 59) to examine fungal and bacterial rRNA genes from small and large intestines (tissue and luminal contents) of restricted-flora (RF) and specific-pathogen-free (SPF) mice. OFRG provides a cost-effective means to extensively analyze microbial community composition. RF mice are descendants of a colony established by antibiotic treatment and inoculation with several Clostridium spp. (13, 17). RF mice possess three immunologic phenotypes: abnormal development of naïve T-cell subsets and regulatory B-cell subsets and the induction of colitis in susceptible mouse strains (13, 16, 17, 62). To our knowledge, this report provides the first culture-independent analysis of fungal rRNA genes from mammalian intestine.

MATERIALS AND METHODS

Mice. SPF C57BL/6 mice bear normal aerobic and anaerobic enteric commensal microflora but are negative for a panel of pathogenic microorganisms based on serologic and microbiologic screening by the UCLA Division of Laboratory Animal Medicine. An RF colony was established 12 years ago in a separate facility maintained by the UCLA Department of Radiation Oncology by extensive antibiotic treatment and recolonization with six species of nonpathogenic Clostridium (13). RF C57BL/6 mice were established 6 years ago by cesarean

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section delivery of SPF fetal C57BL/6 mice and adoptive transfer to RF foster mothers. RF mice were housed in enclosed racks with filtered air and autoclaved bedding, food, and water. For both SPF and RF mice, animals of either gender were used at age 6 to 12 weeks. All animal procedures were performed in accordance with current UCLA institutional review board-approved protocols.

**Intestinal sample collection.** Mice were euthanized by isoflurane inhalation, and the intestines were excised. For DNA extraction, 5- to 10-cm lengths of small intestine or colon were collected, and luminal contents were moved to one end of the intestinal segment with a forceps. Two to 3 cm of the tissue containing the condensed luminal contents was placed in a lysis tube (screw-cap tubes with beads) containing 1 ml CLS-Y buffer from a FastDNA kit (Qiagen, Carlsbad, CA) and immediately frozen at −70°C. For fluorescence in situ hybridization (FISH) samples, small intestine (including jejunum) was harvested and divided into three equally long segments (11 to 12 cm each). In the large intestine, the cecal appendix was excised, and the remaining large bowel was divided into two equal segments (7 to 8 cm each). These tissue samples were divided longitudinally, gently washed with RPMI 1640 ( Gibco, Grand Island, NY) to remove fecal material and luminal debris, fixed at room temperature for 24 h in Carnoy’s solution (15% glacial acetic acid, 85% ethanol), and processed for conventional paraffin embedding.

**DNA extraction from intestinal samples.** Samples in the FastDNA lysis tubes described above were thawed on ice and lysed by bead beating in a FastPrep instrument (Qiagen) for 30 s at setting 5.0. DNA was purified using the FastDNA Kit as described by the manufacturer (Qiagen). DNA was further purified and size fractionated by electrophoresis in 0.6% agarose gels. After staining with ethidium bromide, DNA larger than 3 kb was excised and recovered using the QIAquick gel extraction kit (QIAGEN, Valencia, CA).

**DNA sequence analysis.** DNA templates were digested with the mouse chows (200-ng crushed pellet) using the FastDNA kit and the CLS-Y buffer as described by the manufacturer (Qiagen). DNA was further purified and size fractionated as described above. Details about the chows can be found in Table 4.

**PCR amplification of bacterial and fungal small-subunit rRNA genes.** Bacterial and fungal rRNA genes from small- and large-intestinal samples were obtained by PCR as previously described (60), using 35 amplification cycles. For each sample type, the template was composed of pooled DNA from replicate samples from five mice.

**OFRG analysis.** The compositions of the fungal and bacterial rRNA genes from mouse intestinal samples were obtained by OFRG analyses as previously described (60). Briefly, rRNA gene clone libraries were constructed. rRNA genes from the libraries were then PCR amplified, arrayed on nylon membranes, and hybridized with 32P-labeled DNA probes 10 nucleotides in length. Hybridization signals were used to generate OFRG fingerprints, which were clustered with OFRG fingerprints from taxonomically classified rRNA gene sequences by using the unweighted-pair group method with arithmetic mean. Intestinal rRNA gene clones were categorized by their association with the taxonomically classified rRNA gene sequences and by nucleotide sequence analysis of representative clones distributed throughout the tree determined by the unweighted-pair group method with arithmetic mean.

**rRNA gene analysis of mouse chow.** DNA extracts from the mouse chows were PCR amplified using two sets of universal primers, one targeting all bacterial small-subunit rRNA genes and the other targeting all fungal small-subunit rRNA genes, as described above. rRNA clone libraries were constructed from the resulting amplicons as described above. Nucleotide sequence analysis was performed on 19 randomly selected colonies each from the bacterial and fungal libraries as described below. Two of the bacterial and six of the fungal clones either did not contain an rRNA gene insert or they did not produce reliable nucleotide sequence data and were not analyzed further.

Chow DNA was also analyzed by taxon-selective PCR analyses. Sequence-selective PCR primers were developed for four of the largest assemblages of rRNA gene clones identified by the bacterial and fungal OFRG analyses. Primers were designed by locating DNA sequences that were conserved among the rRNA gene clones within each group by using Pretty (Accelrys, San Diego, CA) and which had few, if any, identical matches to rRNA gene sequences from unrelated libraries as described below. Two of the bacterial and six of the fungal clones as described above. Nucleotide sequence analysis was performed on 19 randomly selected colonies each from the bacterial and fungal libraries as described below. Two of the bacterial and six of the fungal clones either did not contain an rRNA gene insert or they did not produce reliable nucleotide sequence data and were not analyzed further. Chow DNA was also analyzed by taxon-selective PCR analyses. Sequence-selective PCR primers were developed for four of the largest assemblages of rRNA gene clones identified by the bacterial and fungal OFRG analyses. Primers were designed by locating DNA sequences that were conserved among the rRNA gene clones within each group by using Pretty (Accelrys, San Diego, CA) and which had few, if any, identical matches to rRNA gene sequences from unrelated libraries as described below. Two of the bacterial and six of the fungal clones either did not contain an rRNA gene insert or they did not produce reliable nucleotide sequence data and were not analyzed further.

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RESULTS

Composition of intestinal fungal small-subunit rRNA genes.

The compositions of fungal rRNA genes from small and large intestine (tissue and luminal contents) of RF and SPF mice were obtained by OFRG analysis (Table 1 and Fig. 1). Most of the rRNA gene clones were distributed among nine well-defined taxonomic clusters. Two hundred ninety-nine clones were associated with four Ascomycota taxa: Acremonium, Alternaria, Monilinia, and Fusarium. Ninety-six clones were associated with three Basidiomycota taxa: Filobasidium, Cryptococcus, and Scleroderma. Two hundred sixteen clones were associated with two major Chytridiomycota and Zygomycota assemblages: cluster 1 and Mucorales. Four hundred twenty-eight clones had high sequence identity to mammalian rRNA genes. Other rRNA gene sequences identified from small clusters or taxonomically mixed clusters were related to Paecilomyces javanicus, Oxysporum sp., Armillaria borealis, Sordariomycete sp., Galactomyces citri-aurantii, Rhodosporidium toruloides, Plectosphaerella cucumerina, Sordaria fimicola, and Myrothecium verrucaria. The taxonomic identities of the major clusters identified by the OFRG analysis were validated by nucleotide sequence analysis of representative rRNA gene clones. Accession numbers for all of these sequences are listed in "Nucleotide sequence accession numbers" in Materials and Methods. The percent sequence identities to their nearest relatives are shown in Table 1. For several fungal taxa, differences in the number of clones between SPF and RF mice were detected. However, it should be emphasized that OFRG is not a quantitative methodology, so validation of these apparent differences would require additional experimentation.

### TABLE 1. Taxonomic distribution of rRNA gene clones obtained by OFRG analysis of murine intestine with fungus-selective PCR primers

<table>
<thead>
<tr>
<th>Taxon</th>
<th>No. of clones</th>
<th>Nearest relative(s) (accession no.)</th>
<th>% Identity to nearest relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascomycota</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acremonium</td>
<td>477</td>
<td>Acremonium alternatum (AY083232)</td>
<td>99</td>
</tr>
<tr>
<td>Alternaria</td>
<td>7</td>
<td>Alternaria alternata (AF229504)</td>
<td>93–97</td>
</tr>
<tr>
<td>Monilinia</td>
<td>151</td>
<td>Monilinia laxa (Y14210)</td>
<td>96–99</td>
</tr>
<tr>
<td>Fusarium</td>
<td>63</td>
<td>Gibberella pulicaris (AI49875)</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fusarium oxysporum (AI41951)</td>
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<td></td>
<td></td>
<td>Fusarium equiseti (AI41949)</td>
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<td></td>
<td></td>
<td>Fusarium culmorum (AI41948)</td>
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<td></td>
<td></td>
<td>Fusarium cerealis (AI41947)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Gibberella avacaca (AI41946)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cordyceps sinensis (AB067700)</td>
<td></td>
</tr>
<tr>
<td>Unidentified</td>
<td>178</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basidiomycota</td>
<td>154</td>
<td>Filobasidium globisporum (AB075546)</td>
<td>98–99</td>
</tr>
<tr>
<td>Cryptococcus/Filobasidium</td>
<td>51</td>
<td>Filobasidium elegans (AB075545)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cryptococcus magnus (AB032643)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cryptococcus ater (AB032622)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Filobasidium floriforme (D13460)</td>
<td></td>
</tr>
<tr>
<td>Scleroderma</td>
<td>45</td>
<td>Scleroderma citrina (AI026621)</td>
<td>96–97</td>
</tr>
<tr>
<td>Unidentified</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zygomyctota and Chytridiomycota</td>
<td>239</td>
<td>Catenomyces sp. (AY635830)</td>
<td>94–99</td>
</tr>
<tr>
<td>Cluster 1</td>
<td>162</td>
<td>Spizellomyces punctatus (AY546684)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spizellomyces acuminatus (M59759)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neocallimastix sp. (M59761)</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td>Powellomyces sp. (AI64245)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Neocallimastix frontalis (X80341)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Entophlyctis helioformis (AY635826)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mortierella alpina (A271630)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Smittium culisetae (D29950)</td>
<td></td>
</tr>
<tr>
<td>Mucorales</td>
<td>54</td>
<td>Amylomyces rouxii (AY228693)</td>
<td>98–99</td>
</tr>
<tr>
<td>Unidentified</td>
<td>23</td>
<td>Rhizopus oryzae (AI13440)</td>
<td></td>
</tr>
<tr>
<td>Mammalia</td>
<td>428</td>
<td>Backusella ctenidia (AI57122)</td>
<td>98–100</td>
</tr>
<tr>
<td>Unidentified</td>
<td>111</td>
<td>Several mammals, including Mus musculus (BK000964) and Sus scrofa (AY265350)</td>
<td>98–100</td>
</tr>
</tbody>
</table>

* Determined by adding the number of clones in the major taxonomic groups identified by the OFRG analysis (Fig. 1).
* Determined by BLAST (NCBI) (3) analysis of representative clones from the major taxonomic groups identified by the OFRG analysis (Fig. 1). RF-C, colon samples from restricted-flora mice; RF-SI, small intestine samples from restricted-flora mice; SPF-C, colon samples from specific-pathogen-free mice; SPF-SI, small intestine samples from specific-pathogen-free mice.
FIG. 1. Taxonomic depiction of fungal small-subunit rRNA genes identified by OFRG analysis of murine intestine. Major taxonomic groups are indicated. Taxonomic distribution of the rRNA gene clones by mouse and tissue type is listed in Table 1.
FISH analysis of the murine intestine with a universal fungal small-subunit rRNA probe. The OFRG analysis provided evidence suggesting that the murine intestine harbors a diverse array of intestinal fungi. To corroborate these findings, specimens of mouse intestine were subjected to FISH analysis. DAPI staining of the cecum revealed the glandular microanatomy of the mucosa, a thin (0- to 30-μm) mucus layer relatively devoid of organisms, and a luminal biofilm containing organisms and sloughed senescent epithelial cells (Fig. 2a). The organisms in this biofilm were mainly DAPI positive and PF2 negative and were relatively small with rod-like or filamentous morphologies. Previous FISH studies using universal (EUB338) or species-selective small-subunit rRNA probes (55, 56) have shown that most of these microorganisms were of bacterial origin.

When the interlaced layer biofilm was examined by hybridization with a fungus-selective FISH probe (PF2), occasional but distinct PF2-positive microorganisms were observed (Fig. 2a). Morphotypes included filamentous and stout rods, small and large ovoid structures, and very large fusiform structures. These morphotypes were interspersed with each other and the more abundant PF2-negative population. Examples of their morphologies and spatial distribution in different cecal specimens are shown in Fig. 2b to g. These findings were consistent in more than five independent experiments. Only rare DAPI- or PF2-positive microorganisms were detected in cecal crypts or epithelial fields. In three independent experiments, the relative abundance of PF2-positive microorganisms (compared to DAPI-positive microorganisms) in the biofilm was enumerated. The frequency of PF2-positive per DAPI-positive microorganisms ranged from 0 to 10%, with a median of 2%.

Examination of other regions of the intestine revealed much lower levels of DAPI- or PF2-positive microorganisms (data not shown). In the proximal colon, the interlaced layer was colonized with only sparse clusters of DAPI-positive microor-
organisms (~1% of cecal density). Occasional PF2-positive organisms of different morphologies were detected among this population, roughly proportional to the reduced DAPI-positive microbiota. As previously reported, the mucus layer was thicker in the middle and distal colon, with only rare DAPI-positive microorganisms (56); no PF2-positive microorganisms were detectable in this region. DAPI- and PF2-positive organisms were rare in the ileum and undetected in the jejunum.

The fecal compartments of these intestinal segments were also examined by FISH analyses. However, fecal material in the lumen of the intestinal specimens was generally lost during sample preparation. On the occasions where fecal material was present, fungi were detectable in the cecum, rare in the proximal colon, and undetected in the distal colon or expelled fecal pellets. As expected, fecal material itself was unapparent in the ileum. Because of this sampling problem, fungal abundance in the fecal compartment is uncertain.

### Composition of intestinal bacterial small-subunit rRNA genes

The compositions of bacterial rRNA genes from small and large intestines (tissue and luminal contents) of RF and SPF mice were obtained by OFRG analysis (Table 2). Most of the rRNA gene clones were distributed among four well-defined taxa: *Bacteroidetes, Firmicutes, Acinetobacter*, and *Lactobacillus*. The taxonomic identities of the major clusters identified by the OFRG analysis were validated by nucleotide sequence analysis of representative rRNA gene clones. Accession numbers for all of these sequences are listed in “Nucleotide sequence accession numbers” in Materials and Methods. The percent sequence identities to their nearest relatives are shown in Table 2. A striking predominance of *Firmicutes* clones was detected in RF mice, among both the small and large intestinal compartments. Conversely, a more balanced distribution of clones from the predominant bacterial taxa was observed in the SPF mice. As noted above, validation of this conclusion would require additional quantitative experimentation. However, this observation is concordant with the origin of RF mice, which were produced by conventionalization of germfree mice with a mixture of *Clostridium* species.

### rRNA gene analysis of mouse chow

The mouse chows were examined by PCR analyses using primers targeting all bacterial small-subunit rRNA genes, all fungal small-subunit rRNA genes, and four of the largest assemblages of rRNA gene clones identified by the OFRG analyses of the murine intestine. Analysis of the SPF chow with universal bacterial primers identified four corn mitochondrial rRNA gene clones, six rice chloroplast rRNA gene clones, three wheat chloroplast rRNA gene clones, and four different small-subunit rRNA gene clones, none of which were identified in the intestinal analysis (Table 3). Analysis of the SPF chow with universal fungal primers identified one wheat small-subunit rRNA gene clone, one

### Table 2. Taxonomic distribution of rRNA gene clones obtained by OFRG analysis of murine intestine with bacterium-selective PCR primers

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Total</th>
<th>RF-C</th>
<th>RF-SI</th>
<th>SPF-C</th>
<th>SPF-SI</th>
<th>Nearest relative(s) (accession no.)</th>
<th>% Identity to nearest relative(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter</em></td>
<td>313</td>
<td>24</td>
<td>5</td>
<td>145</td>
<td>139</td>
<td><em>Acinetobacter</em> sp. (AJ244765), <em>Acinetobacter</em> sp. (AY588958), <em>Acinetobacter calcoaceticus</em> (AY800383)</td>
<td>99</td>
</tr>
<tr>
<td><em>Bacteroidetes</em></td>
<td>37</td>
<td>5</td>
<td>7</td>
<td>13</td>
<td>12</td>
<td>Uncultured gut bacteria (DQ014784)</td>
<td>99</td>
</tr>
<tr>
<td><em>Firmicutes</em></td>
<td>763</td>
<td>323</td>
<td>305</td>
<td>70</td>
<td>65</td>
<td>Uncultured gut bacteria (DQ014768, DQ015223, AF132269, AF371582, AF371836, AY916380, AY991721, AY991912, AY992417, AY992630, AY992840, AY993011)</td>
<td>99, 94, 91, 93, 98, 91, 96, 96, 95–96, 94, 95, 99, respectively</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>105</td>
<td>0</td>
<td>7</td>
<td>56</td>
<td>42</td>
<td><em>Lactobacillus johnsonii</em> (AE017206)</td>
<td>99</td>
</tr>
<tr>
<td>Unidentified</td>
<td>200</td>
<td>28</td>
<td>54</td>
<td>53</td>
<td>65</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Determined by adding the number of clones in the major taxonomic groups identified by the OFRG analysis (data not shown). RF-C, colon samples from restricted-flora mice; RF-SI, small intestine samples from restricted-flora mice; SPF-C, colon samples from specific-pathogen-free mice; SPF-SI, small intestine samples from specific-pathogen-free mice.

### Table 3. Taxonomic distribution of rRNA gene clones obtained by PCR amplification of DNA extracted from mouse chow using bacterium and fungus-selective primers

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Taxon</th>
<th>No. of clones</th>
<th>Nearest relative (accession no.)</th>
<th>% Identity to nearest relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal bacterial</td>
<td><em>Oryza sativa</em> (chloroplast)</td>
<td>6</td>
<td>AE017114</td>
<td>98–100</td>
</tr>
<tr>
<td></td>
<td><em>Pantoea agglomerans</em></td>
<td>1</td>
<td>AY849936</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td><em>Pantoea</em> sp.</td>
<td>1</td>
<td>AF451269</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas</em> sp.</td>
<td>1</td>
<td>AY131221</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td><em>Triticum aestivum</em> (chloroplast)</td>
<td>3</td>
<td>AJ239003</td>
<td>99–100</td>
</tr>
<tr>
<td></td>
<td>Uncultured bacterium</td>
<td>1</td>
<td>AF371481</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td><em>Zea mays</em> (mitochondrion)</td>
<td>4</td>
<td>AY506529</td>
<td>98–100</td>
</tr>
<tr>
<td>Universal fungal</td>
<td><em>Cladosporium cladosporoides</em></td>
<td>10</td>
<td>AF548071</td>
<td>95–100</td>
</tr>
<tr>
<td></td>
<td><em>Phaeosclera dematioides</em></td>
<td>1</td>
<td>Y11716</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td><em>Setomelanomma hoimii</em></td>
<td>1</td>
<td>AY161121</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td><em>Triticum aestivum</em></td>
<td>1</td>
<td>AJ272181</td>
<td>99</td>
</tr>
</tbody>
</table>

* Chow fed SPF mice.

* Determined by BLAST (NCBI) (3).
Phaeosclera dematioides small-subunit rRNA gene clone, one Setomelanomma holmii small-subunit rRNA gene clone, and 10 Cladosporium cladosporioides small-subunit rRNA gene clones, none of which were identified in the intestinal analyses (Table 3). No PCR products were detected when DNA from the RF chow (which was autoclaved) was amplified with universal bacterial and fungal primers (data not shown). Sequence-selective PCR assays detected Firmicutes, Filobasidium, and Smittium RNA genes in both the RF and SPF chows and Lactobacillus rRNA genes in the RF chow, although some of the amplification reactions did not consistently produce PCR products (Table 4). Analysis of four additional mouse chows detected Filobasidium and Smittium rRNA gene sequences in all four chows and Lactobacillus in three of the chows (Table 4). Analysis of the four additional chows with the Firmicutes-selective PCR assay produced corn mitochondrial rRNA genes (Table 4). Unless otherwise indicated, nucleotide sequence analysis of the PCR products reported in Table 4 confirmed their identities (data not shown).

**DISCUSSION**

Culture-independent analyses of microorganisms inhabiting humans and animals have uncovered a considerable diversity of previously undescribed organisms, the roles of which are just beginning to be understood. Such analyses have been performed for bacterial and viral components of the intestinal microbial community (12, 49, 54). However, to our knowledge, no culture-independent analyses of fungal rRNA genes have been performed on the mammalian intestine. The data presented in this report suggest that the murine intestine, like many other environments (26, 27), contains a diverse array of fungi. OFRG analysis report suggest that the murine intestine, like many other environments, the fungal community (12, 49, 54). However, to our knowledge, no culture-independent analyses of bacterial and fungal components of the intestinal microbiota have been performed (22, 34). As with other culture-independent analyses of bacteria from mice (35, 49), large numbers of bacterial clones identified in this study had relatively low sequence identities to previously described rRNA genes. This result provides additional evidence for the variable and diverse nature of gut bacteria.

**OFRG analysis of intestinal fungi.** OFRG of the mouse intestinal system revealed a diversity of rRNA genes from all major fungal taxa in both the small and large intestines. The Ascomycota sequences were most closely associated with the genera Acremonium, Alternaria, Monilinia, and Fusarium. Acremonium species can form endophytic associations with grass species (52) and cause infections in humans (20). Alternaria alternata is a ubiquitous environmental saprophyte which has been associated with allergic disorders and ocular infections (25, 63). Monilinia laxa has been described as a causal agent of brown rot of stone fruits (21). The form genus Fusarium includes a variety of saprophytes, plant pathogens, human pathogens, and mycotoxin producers (18, 45, 53). The Basidiomycota sequences were most closely associated with the genera Filobasidium, Cryptococcus, and Scleroderma. Members of the genus Scleroderma are found throughout the world and are commonly found on plant tissues (7, 19). Most species appear to be nonpathogenic to humans and animals, with the notable exception of Cryptococcus neoformans.

The majority of the Chytridiomycota and Zygomycota sequences were assembled into two major clusters: cluster 1 and another comprised of members of the order Mucorales. Cluster 1 contains members of the genera Catenomyces, Entophlyctis, Mortierella, Neocallimastix, Powellomyces, Smittium, and Spizellomyces. Catenomyces and Entophlyctis have been identified as inhabitants of aquatic environments (24, 29, 32). Mortierella alpina is a common soil fungus that produces large amounts of arachidonic acid (14, 37). Neocallimastix spp. are anaerobic fungi commonly found in the guts of herbivores (28), and Powellomyces spp. have been identified in soil environments (39). Smittium culisetae is a member of the Trichomycetes, which are...
obligate inhabitants of arthropod guts (36). Although *Tricho-
mycetes* are thought to interact with their host in a commen-
salistic manner, pathogenic behavior of some species has also
been described (36). *Spizellomyces* spp. are commonly found in
soil and can live saprophytically or parasitize fungi and nem-
atoles (40). Sequences belonging to the order *Mucorales*
were related to the genera *Amylomyces*, *Backusella*, and *Rhizopus.
*Amylomyces rouxii* is involved in rice fermentation for the pro-
duction of tape ketan, a traditional Indonesian food (6). *Rhi-
zopus oryzae*, which is also called *Rhizopus arrhizus*, is a patho-
gen of plants and humans (4, 46).

**FISH analysis of intestinal fungi.** FISH analysis showed that
fungi of diverse morphotypes inhabit a poorly organized bio-
film of the cecum and, to a lesser extent, the proximal colon.
Quantitative analysis indicated that fungi form ~2% of the
cecal biofilm microbial population, but their levels were much
lower in the biofilms of other intestinal segments. In fecal
material, fungi were observed in the cecal region, but they were
not detected in the small intestine and the distal colon. Several
analytic issues should be noted. First, there are technical
limitations of FISH analysis. For example, FITC-PF2 hybrid-
ization of cultured *S. cerevisiae* detects only 30 to 50% of
organisms, reflecting factors such as rRNA levels and perme-
abilization efficiency (31). rRNA probes also may fail to detect
certain fungal taxa. Also, fungal taxa may vary in their mor-
phological integrity during intestinal passage or after histologic
processing. If these are important components of the biofilm,
then the relative fungal contribution to the microbial biofilm
may be further underestimated. Second, the intestinal mucosal
biofilm has received only limited study, and these studies have
relied (as in this report) on FISH assessment of microanatomic
sections preserved with Carnoy’s solution (which preserves the
extramucosal mucus layer during histologic processing). The
advent of other recovery or preservation systems would valid-
ate and possibly refine biofilm scale and composition.

**Role of intestinal fungi.** The discovery of these fungal rRNA
genes raises important functional questions. What role, if any,
do fungi play in food metabolism for nutrient and energy
bioavailability? Since anaerobe fungi contribute to the diges-
tion of fibrous materials in ruminants (1, 23), they may play a
similar role in nonruminant animals. Similarly, how might fungi
contribute to microbial immune homeostasis? Several
recent reports have implicated fungi in the development of
allergic and immune responses. In mice, antibiotic perturba-
tions of gastrointestinal communities coupled with *Candida
albicans* supplementation led to an increase in levels of eosino-
philic, mast cells, interleukin-5, interleukin-13, gamma interferon,
immunoglobulin E, and mucus-secreting cells in response to ex-
posure to *Aspergillus fumigatus* (44). The authors of that study
suggest that this response may be caused by the production of
prostaglandin-like oxylipins by the fungus. In addition, as fungi
produce an assortment of immune-modulating compounds, it has
also been suggested that these organisms could be associated with
a range of immune alterations (43). These findings, coupled with
the diverse array of rRNA genes identified in this study, suggest
that fungi may play an important role in microbial immune ho-
meostasis.

**Microbial analysis of mouse chow.** Analysis of the mouse
chow suggested that some of the microbial rRNA genes iden-
tified in the intestinal analysis, and/or the organisms repre-
sented by these genes, were present in the chow. When assays
targeting some of these specific rRNA sequences were used,
most of them were detected in the chow. However, when assays
targeting all bacteria and fungi were used, none of the rRNA
genes identified by analysis of the intestine were detected in
the chow. Given that the universal bacterial primers anneal to
corn mitochondrial, rice chloroplast, and wheat chloroplast
rRNA genes with either one or no mismatches, it is not sur-
prising that these plant-derived sequences predominated this
analysis. Taken together, these results suggest that some of the
intestinal rRNA genes were present in the chow but that they
were a minor constituent compared to the plant components.

Interpreting the data from this study is complicated by the
detection of some of the same microbial rRNA genes in both
the intestine and chow. The rRNA genes identified in the
intestinal samples may have come from intestinal inhabitants,
or they may have originated from the chow in the form of living
organisms, dead organisms, or their DNA. Although distin-
guishing among these possibilities is not a simple task, it may
be an important one. For example, in a study examining the
probiotic bacterium VSL-3, the viable bacterium and its DNA
attenuated colitis, while the heat-killed bacterium did not (47).
At this point, however, it is unclear how such studies could be
performed on complex communities of intestinal microorgan-
isms, many of which are yet to be described.

Detection of some of the same microbial rRNA genes in
both the intestine and chow could also influence the design of
future experimentation as well as the interpretation of data
from past and future investigations. Experimental design con-
siderations derived from these results should include chow
type selection. For example, when comparing germfree and
conventionally raised animals fed different chows, chow type
should be considered a potential variable. Concerning data
analysis, when interpreting results from rRNA gene analysis of
intestine or feces, it may be important to consider the origin of
the sequences.

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